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Optimization of extraction and PCR amplification of RNA extracts from paraffin-embedded tissue in different fixatives

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Summary

A method was developed for fast and efficient isolation of RNA from paraffin-embedded tissue sections for subsequent PCR analysis. This method is based on the binding of RNA to acid-treated glass beads in the presence of a high molarity of guanidinium salt. It can be completed within an hour, and obviates the need for dewaxing and phenol/chloroform extractions. The effect of various fixatives and fixation times was tested and the amplification of actin mRNA fragments ranging in length from 82 to 507 bp was used to demonstrate the presence of RNA in the extracts. The method was compared to existing extraction techniques by studying the quality of the templates for reverse-transcriptase polymerase chain reaction amplification (RT-PCR), using virus-infected and mock-infected paraffin-embedded cell pellets as a model. PCR amplification of cellular and viral RNA was successful for RNA isolated by use of all extraction techniques, although the glass bead method was preferred for its simplicity and rapidity. Specimens fixed with formalin were found to be suitable for PCR, but the best results were obtained with acetone-fixed paraffin-embedded material. Dewaxing of tissue sections had no effect on the yield and quality of RNA extractions, and further purification of the extracts using gel filtration did not improve the results. After the protocols were optimized, rotavirus-infected cell pellets were used to demonstrate that

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extraction and amplification of dsRNA was possible. The information obtained from the studies with the model system was used for extraction of toroviral and rotaviral RNA from archival intestinal material. These data indicate that paraffin-embedded archival tissue can be used for RT-PCR analysis, adding an important technique to diagnostic pathology and retrospective studies.

Viral RT-PCR; Paraffin; Fixatives; Extraction method

Introduction

In pathology the analysis of RNA by molecular methods is often hampered by the lack of fresh material, making diagnostic and retrospective studies difficult, if not impossible. Pathology departments accumulate large numbers of paraffin-embedded tissues, dating back many years. The extraction of DNA from such specimens for polymerase chain reaction (PCR) analysis has been well documented (Forsthoevel et al., 1992; Greer et al., 1991; Cuzick et al., 1992; Heller et al., 1991; Jackson et al., 1990; Lai-Goldman et al., 1988). However, relatively little is known about the application of reverse-transcriptase (RT)-PCR to detect cellular or viral RNA in paraffin-embedded tissue and about the influence of different fixatives on RT-PCR (Redline et al., 1991; Rupp and Locker, 1988; Stanta and Schneider, 1991). A previously described glass bead method that we used for RNA extraction from stools (Boom et al., 1990; Koopmans et al., 1991b) was modified and used to extract RNA from paraffin-embedded specimens. This method was compared to existing methods (Rupp and Locker, 1988; Stanta and Schneider, 1991). In doing so, the practical limitations of these approaches were addressed. We studied the effect of fixatives and RNA extraction technique on subsequent PCR in an in vitro system. Toroviruses provided a good model system, because: (1) they have a single stranded RNA genome (Snijder et al., 1988; Weiss et al., 1983). Single-stranded RNA is highly susceptible to degradation by RNAses, and our hypothesis was that, if we could extract intact ssRNA, the application of the techniques for extraction of dsRNA would be relatively easy; (2) they have been adapted to cell culture (Weiss et al., 1983), enabling us to use infected cell pellets as a model; (3) they have been partially sequenced and diagnostic RT-PCR assays were established in our laboratory (Koopmans et al., 1991a and 1991b; Snijder et al., 1989, 1990a, 1990b); (4) tissues from experimentally infected calves were available to apply the techniques on clinical specimens (Fagerland et al., 1986; Pohlenz et al., 1984).

The equine torovirus, Berne virus (BEV), replicates in equine dermis cells or equine mule skin cells, where it causes a cytopathic effect that results in cell lysis (Weiss et al., 1983). It is believed to be non-pathogenic, but is the only torovirus that has been isolated in cell culture and serves as the prototype (Weiss and

Horzinek, 1987). The toroviruses that cause diarrhea in calves (Breda virus type 1 and 2; Koopmans et al., 1991c; Woode et al., 1982) cannot be propagated outside of the natural host, which has hampered detailed characterization of these viruses. However, limited nucleotide sequence analysis has shown that the 3′-non-coding regions of BEV and Breda virus are highly conserved (96% identity; Koopmans et al., 1991b) and we used this region for PCR amplification. Additional PCR primer sequences were chosen from a region in the polymerase protein gene that is conserved between toroviruses and coronaviruses (Snijder et al., 1990b).

In the work reported here we used paraffin-embedded torovirus-infected cell pellets to compare the effect of different fixatives and RNA extraction methods on RT-PCR amplification of tissue extracts. The optimal methods were subsequently tested for extracting dsRNA using rotavirus-infected and uninfected cell pellets. Finally, we report the successful application of our findings to clinical specimens.

Methods

Preparation of paraffin-embedded cell pellets

Berne virus strain P138/72 was propagated in Equine Dermis cells P20-30 (E. Derm, ATCC CCL 57) in Hepes (20 mM) buffered Eagle's Minimal Essential Medium with 10 mM non-essential amino acids, 2 mM glutamine, 10% fetal calf serum, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate (Weiss et al., 1983). Confluent monolayers were infected with BEV at multiplicity of infection 0.01 and trypsinized after 19 h at 37°C. The cells were pelleted by low-speed centrifugation, washed with 0.01 M phosphate buffered saline solution (PBSS) pH 7.4, aliquoted into 15 ml Falcon tubes at 2 $\times$ 10^7 cells per pellet and fixed in 10% neutral buffered formalin (NBF; 6 h, 24 h, 5 mo), 4% paraformaldehyde in PBSS (PFA, 30′), Carnoy's (2 h), citrate buffered acetone (CBA, 5′) or B-5 fixative (2 h) (Sheehan and Hrapchak, 1980). After fixation the pellets were washed with diethyl pyrocarbonate (DEPC) treated PBSS and stored in 70% ethanol before embedding.

Rotavirus strain Wa was grown in MA104 cells (Biological Products Branch, CDC, Atlanta, GA) in medium 199 (GIBCO, Long Island, NY) with 5% fetal calf serum and antibiotics. After overnight incubation the cells were harvested and processed as described above.

Clinical specimens

For the torovirus RT-PCR 8-yr-old tissue sections from the anterior and posterior jejunum of a gnotobiotic calf (kindly provided by Dr. G.N. Woode) were extracted as described below in protocol B. Full experimental details on this calf (GC66) have been reported previously (Fagerland et al., 1986). Briefly,

the calf had been infected at 24 h of age with bovine torovirus and intestinal tissues were removed from the upper and lower jejunum under anesthesia at 26 h post-infection; the calf was then killed. The tissues were formalin or acetone fixed. By electron microscopy and immunofluorescence staining, torovirus-infected cells were found sporadically in the epithelium of the anterior jejunum; by contrast the majority of the epithelium of the posterior jejunum was infected (Pohlenz et al., 1984).

Formalin-fixed and paraffin-embedded intestinal tissue from 4 children were obtained from the International Center for Diarrheal Disease Research in Dhaka, Bangladesh. The tissues had been selected from archival materials based on the postmortem findings suggesting death as a result of diarrhea. No premortem rotavirus test results were available.

Oligonucleotides

The sequences of the oligonucleotides used for torovirus and β-actin RT-PCR are listed in Table 1. The primers actin 1 and 5 were based on sequences that have been published previously (Brenner et al., 1989). The other primers were selected using the computer program 'OLIGO' (Rychlik and Rhoads, 1989). They were synthesized at the Biotechnology Core Facility, Centers for Disease Control and Prevention, Atlanta, GA. Primer combinations actin 1/3, actin 1/4, and actin 1/5 were used to amplify β-actin mRNA fragments of 82, 249 and 507 bp, which can be distinguished from the actin gene amplification products by their smaller sizes due to lack of an intron. Nevertheless, DNA contamination of the templates for β-actin RT-PCR may result in false-positive results since a processed actin pseudogene

TABLE 1

Sequences of the primers that were used for the torovirus and β-actin RT-PCRs described in Materials and Methods.

Primers	Sequence
toro 1	5′taa aac ctt ttc agc aac ctt 3′
toro 2	ttt ttt ttt ttt agc tgc ttt t
toro 3	aat gat ttt caa gat gta cag ttt atg
toro 4	ctt cac atg atc ttg aat aaa ggt aca
actin 1	gtg ggg cgc ccc agg cac ca
actin 2	ggg cat ggg tca gaa gga ttc cta
actin 3	ctc ttg ctc tgg gcc tcg tc
actin 4	tgg gtc atc ttc tcg cgg tt
actin 5	ctc ctt aat gtc acg cac gat ttc
Primer pair	**Predicted product size**
toro 1/2	231 bp
toro 3/4	235 bp
actin 1/3	82 bp
actin 1/4	249 bp
actin 1/5	507 bp

exists (Zaki et al., submitted for publication) and therefore must be avoided.

RNA extraction

Three 8-μm sections of paraffin-embedded formalin-fixed cell pellets were cut using disposable microtome blades and placed in sterile 1.5 ml microcentrifugation tubes. RNA was extracted either directly or after deparaffinization, which was done by rinsing with Pro-par (xylene substitute, Anatech Ltd., Battle Creek, MI), followed by 2 ethanol washes and drying of the pellets under vacuum. Tissues were incubated for 30′ at 45°C in 1 ml of a lysis buffer containing 4.7 M guanidinium isothiocyanate (GuSCN; Boehringer Mannheim, Indianapolis, IN), 20 mM ethylene-diamine-tetra-acetate (EDTA), 100 mM Tris pH 6.4 and 1% Triton X-100. RNA was bound to size-fractionated silicon dioxide (SC; Sigma Chemical Company, St. Louis, MO) particles by adding 50 μl of SC to the samples and incubating them for 40′ on a rocking table at RT (Boom et al., 1990; Koopmans et al., 1991b). The SC particles were pelleted by centrifugation in a tabletop centrifuge for 10 s at 10000 $\times$ *g*, and then washed twice with 1 ml GuSCN wash buffer (4.7 M GuSCN, 100 mM Tris, pH 6.4), twice with 1 ml of 70% ethanol and once with 1 ml of acetone. After removal of the acetone, the pellets were dried and nucleic acids were eluted off the beads at 70°C for 15′ in 100 μl of DEPC-treated water with 1 mM dithiothreitol (DTT) and 0.5 U/μl RNAse inhibitor (Human Placenta RNAse Inhibitor; Boehringer Mannheim, Indianapolis, IN).

To study the effect of further purification, aliquots of each eluate were extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) after adding 1/10th volume of 3 M sodium acetate (pH 5.2) followed by precipitation with 2 volumes of ethanol. The pellets were resuspended in DEPC-treated distilled water, containing 1 mM DTT and 0.5 U/μl RNAse inhibitor. Half of the samples were further purified using RNAse-free G50 Sephadex columns (Quick Spin™ columns; Boehringer Mannheim Biochemicals, Indianapolis, IN).

The performance of this method was compared to 2 other RNA methods using proteinase K treatment in the presence (protocol A) or absence (protocol B) of GuSCN.

Protocol A

RNA was extracted by proteinase K digestion in the presence of guanidinium isothiocyanate as described by Stanta and Schneider (1991). Briefly, the sections were digested in 200 μl of 6 mg/ml proteinase K, 1 M GuSCN, 25 mM β-mercaptoethanol, 0.5% Sarkosyl™, 20 mM Tris, pH 7.5, for 6 h at 45°C. After inactivation of the proteinase for 7′ at 100°C, the digests were further purified by phenol/chloroform extraction, ethanol precipitation and column purification as described.

Protocol B

RNA was obtained by digesting the sections for 6 h at 45°C in 450 μl proteinase K digestion buffer containing 10 mM NaCl 500 mM Tris, pH 7.6, 20 mM EDTA, 1% sodium dodecyl sulfate and 0.5 mg/ml proteinase K (Rupp and Locker, 1988), followed by heat inactivation of proteinase K, phenol/chloroform extraction, ethanol precipitation and spin column purification as above.

The RNA extraction methods were evaluated by testing aliquots that were taken before each phenol/chloroform extraction and after each ethanol precipitation by torovirus and actin RT-PCR as described. The purification process was monitored by spectrophotometry at 260 and 280 nm.

Comparison of fixatives

For this comparison, cell pellets that had been fixed with formalin, PFA, Carnoy's, CBA and B-5 as described were extracted using the SC method and protocols A and B without column purification. The extracts were tested by torovirus and actin RT-PCR. A summary of the experimental design is shown in the flow chart (Fig. 1).

RT-PCR

We used three different RT-PCR systems, assaying for the presence of actin mRNA and torovirus genomic and mRNA. The actin RT-PCR served as a control for the presence of intact RNA. One torovirus primer pair was chosen from the 3′-end of the equine torovirus sequence, which according to computer modelling is folded into a mostly double-stranded structure. A second pair of primers was chosen from the toroviral polymerase protein gene, for which no such secondary structure has been predicted, rendering it more sensitive to RNAse degradation. Negative control reactions (distilled water and extracts from uninfected cells) were routinely included. To avoid false positives, all RT-PCR preparations were carried out in a different place from that of the postamplification manipulations with a different set of pipets. Also, paraffin-embedded cell pellets were prepared and tissue sections cut in a different part of the building, and a new disposable microtome blade was used for each block.

Actin RT-PCR Actin mRNA was chosen as a reporter target for amplification to monitor the presence of cellular RNA, assuming that negative amplification results indicate RNA degradation or inhibition of RT-PCR, or both. We used 3 primer pairs (Table 1) that form a nested set of amplicons of 85, 249 and 507 bp with both human and equine mRNA templates. To avoid DNA amplification that might occur due to the presence of a processed pseudogene (Zaki et al., submitted for publication) and/or RT activity of Taq polymerase (Jones and Foulkes, 1989; Tse and Forget, 1990), the extracts were incubated prior to reverse transcription with 0.1 U/μl of

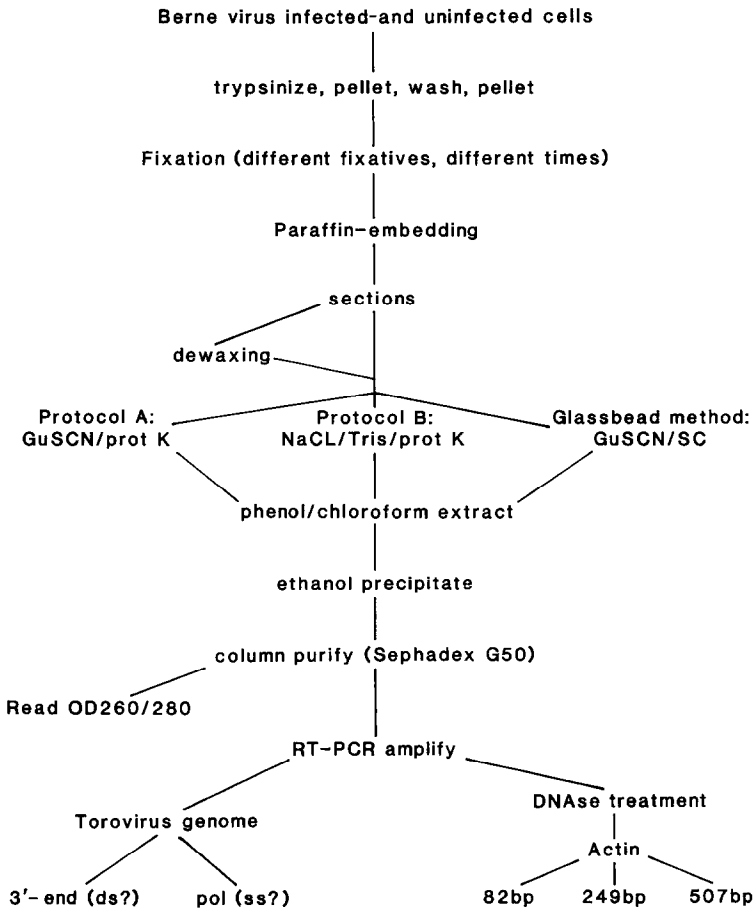

Fig. 1. Flow chart of experiments described in the 'Methods' section.

RNAse-free DNAse (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 1 h at RT, followed by heat inactivation of the enzyme for 5′ at 70°C. Plasmid DNA containing the 3′-end region of BEV (pBSN, Snijder et al., 1989) was digested and amplified in parallel with untreated DNA to see whether digestion was complete. The RT-step was done in a 50 μl final volume containing 1 × PCR buffer (50 mM Tris-HCl, pH 8.3, 10 mM KCl, 1.5 mM $MgCl_2$), with 0.4 mM deoxynucleoside triphosphates, 1 mM DTT, 25 pmole antisense primer and 10 U avian myeloblastosis virus RT (Boehringer Mannheim, Indianapolis, IN). After 1 h at 42°C 50 μl of 1 × PCR buffer with 25 pmol of the sense primer and 2.5 U Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT, USA) were added. The reaction mixture was incubated for 40 cycles of 1′ at 94°C, 1′ at 43°C and 1′ at 72°C, followed by a final extension of 7′.

Torovirus RT-PCR, 3'-end The primer sequences (toro 1 and 2, Table 1) were chosen from domains at the 3'-non coding region of the torovirus genome that are fully conserved between bovine and equine toroviruses (Koopmans et al., 1991b). Their sensitivity and specificity were assayed by using RNA extracted from cell culture-grown BEV as positive control and RNA extracts from uninfected cells as well as from cell culture-grown coronavirus as negative controls (mouse hepatitis virus and human respiratory coronavirus OC43, kindly provided by Dr. C. Stephensen, University of Alabama, Birmingham). The RNA was mixed with 50 pmol primer toro 2, denatured for 5' at 70°C and cooled on ice as a separate annealing step gave slightly better results with this primer-template combination. The RT and PCR were done as described above in 1 × PCR buffer containing 2 mM $MgCl_2$, 1 mM dNTPs and 50 pmol of each primer. The reaction mixture was incubated for 30 or 40 cycles of 1' at 94°C, 1' at 45°C and 1' at 72°C, followed by a final extension of 7'.

Torovirus RT-PCR, polymerase region The primer sequences (toro 3 and 4, Table 1) were chosen from a region in the polymerase gene that is conserved between toroviruses and coronaviruses as shown by Snijder et al. (1990b). The RT-PCR was performed as described above, but at a final $MgCl_2$ concentration of 2.5 mM.

Rotavirus RT-PCR

Rotavirus RT-PCR was done by Dr. J. Gentsch at the rotavirus laboratory of the Viral Gastroenteritis Section, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta GA, USA, as described previously (Gentsch et al., 1992). The primer pair Con 1/2 (amplicon size 211 bp; Gentsch et al., 1992) was used.

Gel analysis and Southern blots of RT-PCR products

The products were run on agarose (1.5–3% in 0.04 M Tris-acetate, 1 mM EDTA) or polyacrylamide gels (8% in 0.09 M Tris-borate, 2 mM EDTA) and stained with 0.5 μg/ml ethidiumbromide in H_2O.

Products of actin RT-PCRs were transferred to a nylon membrane (Nitroplus 2000, Micron Separations, Westboro, MA) by Southern blotting (Southern, 1975) and hybridized to ^{32}P-labelled oligonucleotide actin 2 (Table 1) under stringent conditions, essentially as described previously (Judd et al., 1991).

Results

Comparison of extraction methods

In a pilot experiment, formalin-fixed, paraffin-embedded cells were used to compare the 3 extraction protocols. Optical density readings that we used to monitor the purification process showed a gradual increase in 260/280 ratios after the phenol/chloroform extraction and column purification steps in each protocol, as expected, but they never reached levels indicating high purity of RNA (1.6 maximum, pure RNA 2.1). There were no significant differences between the extraction procedures, and dewaxing did not give significantly better results, although the ratios tended to be slightly higher than those of extracts from non-dewaxed tissue sections.

In Fig. 2a the results of RT-PCR amplification are shown, using the

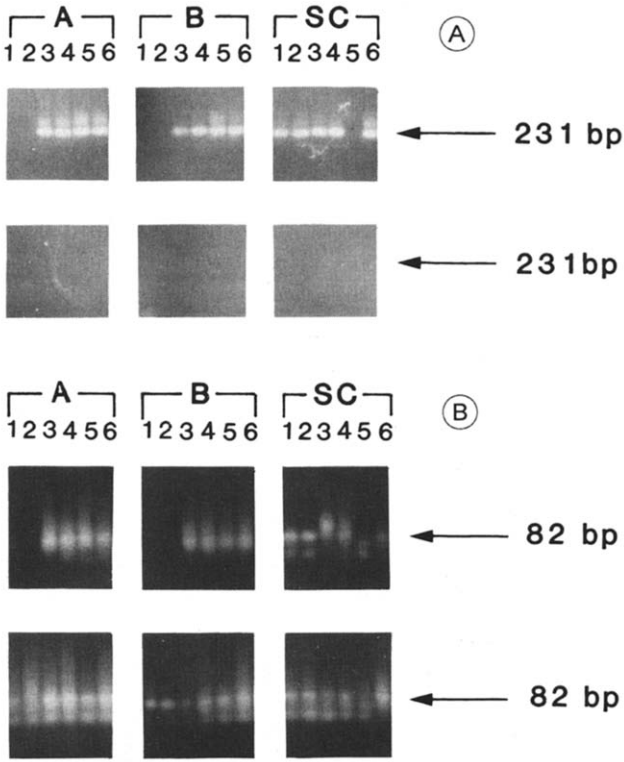

Fig. 2. Results of RT-PCR amplification using primers toro 1 and 2 (2a), and actin 1 and 3 (2b). RNA was extracted from formalin-fixed, paraffin-embedded torovirus-infected (2a, upper panel) or uninfected (2a, lower panel) E. Dermis cells, according to protocol A, B or the glassbead method (see text) without (odd numbers) or with (even numbers) prior dewaxing. Templates for the RT-PCR reactions were aliquots taken during the RNA extractions before (2a, lanes 1 and 2) and after (lanes 3 and 4) phenol/chloroform treatment, and after the spin columns (lanes 5 and 6). The β-actin RT-PCR was done with 10 μl (2b, upper panel) or 1 μl (lower panel) of template per reaction.

torovirus 3′-end primers (40 cycles). Amplifiable RNA was obtained with all 3 extraction methods. In protocols A and B phenol/chloroform was necessary for successful amplification, because only after such treatment were visible bands obtained (A3-6, B3-6). Spin column purification did not give better RT-PCR results (reactions 5 and 6) than phenol/chloroform extraction alone (reactions 3 and 4), and lead to the loss of one template (C5). Finally, RT-PCR results were comparable for extracts from dewaxed and non-dewaxed sections. Identical results were obtained when using torovirus primers 3 and 4 (not shown).

With actin primers 1 and 2, which give rise to an amplicon of 82 bp, results were similar to the torovirus RT-PCR when 10 μl extract was used (Fig. 2b, top panel). No amplification was obtained with aliquots from extracts that were taken prior to phenol/chloroform/isoamylalcohol extraction in protocols A and B (lanes A1, A2, B1 and B2). When a 10-fold dilution of these templates was used in the RT-PCR, clear bands were seen (Fig. 2b, lower panel),

TABLE 2a

Results of torovirus 3′-end and polymerase gene RT-PCR amplification for different fixatives and extraction methods

Fixative	Protocol A		Protocol B		Glassbead	
	3′-end	pol	3′-end	pol	3′-end	pol
Formalin 6 h	–	–	+ +	+ +	+ +	+
Formalin 24 h	+ +	–	+ + +	+	+	–
Formalin 5 mo	–	–	–	–	–	–
PFA	+	+	+ + +	+ +	+ +	+ +
Carnoy's	–	+	+ +	+	–	–
CBA	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
B-5	+ +	+ + +	+ + +	+ + +	+ + +	+ + +

Phenol/chloroform extractions and ethanol precipitation were done with protocols A and B, but not with the glassbead (SC) method. Band intensity was scored visually in comparison with the DNA marker as weaker (+), similar (+ +) or stronger (+ + +). Abbreviations: PFA, paraformaldehyde; CBA, citrate-buffered-acetone; pol, polymerase.

TABLE 2b

Results of PCR amplification of actin genes and mRNA for different fixatives and extraction methods using primer pairs capable of amplifying 82, 249 and 507 bp, respectively

Fixative	Protocol A	Protocol B	Glassbead
	RNA	RNA	RNA
Formalin 6 h	82	82	82
Formalin 24 h	82	82	82
Formalin 5 mo	82*	82*	82*
PFA	82	82	82
Carnoy's	82	82	82
CBA	82	507	249
B5	82	82	82

The maximum size of amplicon obtained with the three primer-pairs is given.
*indicates PCR bands could only be detected by hybridization with an internal oligonucleotide.

indicating that the lack of amplification had been caused by inhibition of RT-PCR rather than shortage of template.

Effect of fixatives and extraction method on RT-PCR

The results of this experiment are listed in Tables 2a and 2b. For this comparison we extracted sections from paraffin-embedded cell pellets that had been fixed for different times in different fixatives. Extraction protocols A and B and the glass bead extraction method were used, and RT-PCR amplification was done using primer pairs toro 1/2 (3-end), 3/4 (pol; Table 2a), actin 1/3 (82 bp), 1/4 (249 bp) and 1/5 (507 bp; Table 2b). For the torovirus RT-PCRs, less template and a lower number of cycles (30) were used than in the comparison of extraction methods described above (40 cycles) in order to detect differences in efficiency of extraction and RT-PCR. When using the 3′-end torovirus primers (1/2) under these conditions, most consistent results were obtained with extraction protocol B (Table 2a), which yielded amplifiable RNA from cell pellets for all fixations except 5 months formalin prior to embedding. It is noteworthy that we were able to amplify an 82 bp β-actin RNA fragment from the latter tissue (primers actin 1 and 2; Table 2b). For materials fixed in Carnoy's fluid, only protocol B gave positive RT-PCR results with both torovirus primer pairs (Table 2a). With the polymerase primers, toro 3 and 4, the results were similar but the intensity of the DNA bands was less for 4 of the 14 extracts that gave positive results. To compare the sensitivities of the 2 torovirus specific primer pairs, we tested RNA extracts from serially diluted cell-culture grown BEV, showing that the 3′-end primers detected 10 and the polymerase primers 10^2 TCID50 (not shown). No bands were obtained when coronavirus RNA was used as a template.

When comparing fixation methods, the best results were obtained with acetone and B-5 as a fixative, which gave high yields of torovirus PCR product with all extraction protocols. However, the maximum size of amplifiable actin mRNA was higher for the acetone-fixed material (Table 2b). Even though the size of the torovirus amplicons was 231 for the 3′-end primers and 235 for the polymerase primers, the similar size (249 bp) product of the actin RT-PCR (primers actin 1 and 3) was only seen in 4 of the 13 extracts that were positive with both torovirus primers.

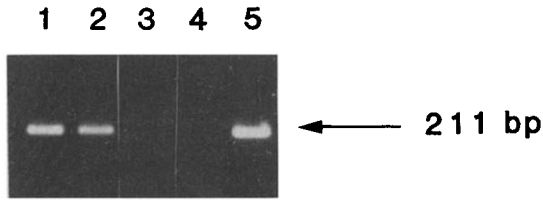

Fig. 3. Rotavirus RT-PCR was performed on RNA extracted from infected (lanes 1 and 2) or uninfected (lane 3) formalin-fixed, paraffin-embedded cells. RNA extracts from fresh lysates of uninfected (lane 4) or rotavirus-infected (lane 5) MA104 cells were used as negative and positive controls, respectively.

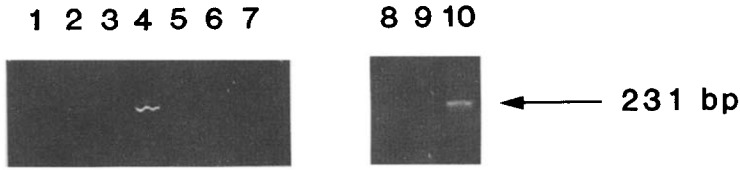

Fig. 4. Results of RT-PCR amplification of extracts from archival specimens from a calf experimentally infected with bovine torovirus. Template RNA was extracted from formalin (1 and 2) or acetone-fixed (3 and 4) sections of the anterior (1 and 3) and posterior (2 and 4) jejunum. RNA extracted from tissues of an uninfected gnotobiotic calf (5 and 6), as well as fresh RNA extracts from uninfected (lane 9) and infected (lane 10) Equine Dermis cells were used as controls. Lanes 7 and 8 contain master mix controls.

To test if our method was applicable to rotavirus, which has a double-stranded RNA genome, we extracted RNA for RT-PCR from paraffin-embedded rotavirus-infected and uninfected cell pellets (Fig. 3, lanes 1–3). We used NBF as a fixative in this experiment because we had archival pathology specimens to be tested for rotavirus, that were formalin-fixed. RNA from fresh uninfected and infected cells was used in the negative and positive control reactions (Fig. 3, lanes 4 and 5).

Amplification of viral RNA from archival specimens

In Fig. 4 the results are shown for RT-PCR using torovirus 3′-end primers and RNA that had been extracted from formalin- (lanes 1 and 2) or acetone-fixed (lanes 3 and 4) tissue sections from the anterior (lanes 1 and 3) and posterior (lanes 2 and 4) jejunum of a torovirus-infected experimental calf. Amplification products were obtained with both fixatives (product in lane 2 not visible) but the bands were stronger for the acetone-fixed material. Only extracts from sections of the posterior jejunum resulted in a positive RT-PCR. RNA from uninfected calf intestine gave no amplification products (lanes 5 and 6).

Five intestinal tissues from 4 children that had died from diarrhea in Dhaka, Bangladesh, were extracted and assayed by RT-PCR (Fig. 5, lanes 1–5). Two of the samples (lanes 1 and 3) showed a clear band, one of them (lane 5) a faint band. Two samples were negative (lanes 2 and 4).

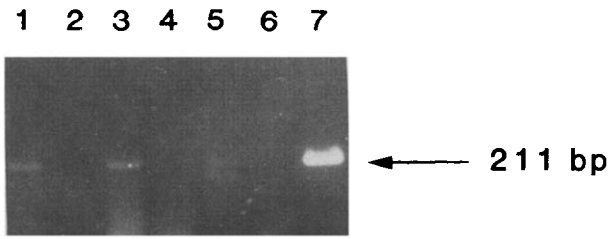

Fig. 5. Results of RT-PCR amplification of extracts from formalin-fixed paraffin-embedded intestinal tissues from children that died from diarrhea (lanes 1–5). Negative (lane 6) and positive (lane 7) controls were as described in the legend of Fig. 3.

Discussion

The use of a model system has permitted the evaluation of different RNA extraction techniques for paraffin-embedded material and the influence of type and time of fixation prior to embedding. The use of torovirus as a model system enabled us to study their applicability on pathology specimens.

We tried to monitor the quality and quantity of RNA obtained with each of the extraction methods by spectrophotometry. However, we found that only a very crude quality indication could be obtained from the 260/280 ratios which generally were low, even after spin column purification. We were unable to find a relation between the optical density readings and ratios and the results of subsequent RT-PCR amplification. Therefore, we refrained from further use of this method. RT-PCR amplification of actin RNA using primers 1 and 3 (82 bp product) was a certain indication of the presence of amplifiable RNA in the extracts but was more sensitive than the torovirus-specific RT-PCRs (Tables 2a and b). Interestingly, a 249 bp actin-specific amplicon was only obtained in 2 of the 13 extracts that were positive in both torovirus RT-PCRs giving a similar size product. This suggests that the viral RNA was less prone to degradation. Alternatively, this primer pair may have a lower sensitivity than either pair of torovirus primers.

The actin primers were primarily used for the model system and give an indication whether intact RNA is present. In clinical specimens other reporter RNAs may be more appropriate, especially in tissues that contain cells of different durability. This is the case in intestinal sections because the mucosa cells slough off into the lumen within 30′ postmortem. Positive actin RT-PCR may result from extraction of the submucosal layers, thus falsely suggesting integrity of the cells that are relevant to the diagnosis of viral infection. The use of primers specific to mucosa cell RNAs (e.g. villin; Arpin et al., 1988; Bazari et al., 1988) should be evaluated.

All 3 protocols gave amplifiable RNA when sufficient template was used for the PCR. The simplicity and low risk of contamination of the SC method makes it the method of choice for extraction of abundant nucleic acids. Since spin column purification did not improve the results in our hands and increases the risk of contamination and RNA loss, we omitted this step after the first extractions. Similarly, since prior dewaxing did not seem to influence the results we omitted that step, thus further decreasing the risk of cross-contamination. A modification of extraction protocol B gave the best overall results when we lowered the amounts of templates. Our results suggest that in general there is no advantage provided by additional purification of RNA extracted by spin column purification. However, this may be dependent on the efficiency of the primers used in a study, and amplification with degenerate or inefficient primers may be enhanced by further purification of the target RNA.

One of our goals was to establish a guide for tissue fixation procedures that could be useful for designing retrospective and prospective PCR studies. With all protocols, acetone fixation clearly gave the best quality and quantity of

RNA template. Neutral buffered formalin, which is the most commonly used fixative, did result in sufficient RNA, but fixation time and extraction method were important. After 6 or 24 h of fixation, intact RNA could be obtained, whereas after 5 months, only weak bands were seen after amplification with the primer pair giving the smallest size amplicon (actin 1 and 3; 82 bp). We have used varying times of fixation outside of this study and had similar results up to 1 wk of fixation before embedding, with decreasing RNA yields thereafter. These results are in agreement with those of Rupp and Locker (1988), who used Northern blot hybridizations to demonstrate progressive degradation of RNA in tissues fixed and stored in NBF. Singer et al. (1986) found bottled formalin solutions to be unpredictable in their effect on RNA retention in tissues to be tested by in situ hybridization. They also showed that excellent RNA retention was obtained with a mercury fixative similar to B-5, in agreement with our findings, and PFA. Acetone was not used in their study, and it would be interesting to test whether acetone-fixed material is equally suited for in situ hybridization and PCR.

The successful amplification of rotaviral and toroviral RNA from archival specimens confirmed the results obtained in the model system and demonstrated the clinical applicability. The torovirus RT-PCR results correlated well with the findings in a study by Fagerland et al. (1986), who found numerous torovirus-infected cells in the intestinal epithelium of the posterior jejunum (which tested positive by RT-PCR) and not in the anterior jejunum, which was negative when tested by RT-PCR. The importance of the fixative used, however, is clearly demonstrated by the difference in intensity of the RT-PCR products from extracts of formalin-fixed and acetone-fixed material.

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